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PCR-based capsular serotype determination of *Haemophilus influenzae* strains recovered from Japanese paediatric patients with invasive infection

N. Ishiwada, L. D. Cao and Y. Kohno

Department of Paediatrics, Graduate School of Medicine, Chiba University, Chiba, Chuo-ku, Inohana, Japan

ABSTRACT

The serotypes of 53 isolates of *Haemophilus influenzae* from children with invasive infections were determined by a conventional slide agglutination test (SAT) and a recently proposed PCR-based method for serotyping *H. influenzae*. The PCR assay identified 47 (88.7%) type b isolates, one (1.9%) type e isolate and five (9.4%) non-typeable isolates. The only discrepancy between the methods was an isolate that was non-typeable by SAT, but was identified as serotype e by PCR. Of 41 isolates from patients with meningitis, 39 (95.1%) were type b. Of the five non-typeable isolates, three (60%) were from the blood of patients with septicaemic pneumonia and two (40%) were from the cerebrospinal fluid of patients with meningitis. None of the non-typeable isolates appeared to be a capsule-deficient mutant of an encapsulated *H. influenzae* strain. Overall, the study confirmed the usefulness of this PCR method for the serotyping of invasive *H. influenzae* isolates.

Keywords *Haemophilus influenzae*, meningitis, PCR, serotyping

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INTRODUCTION

Haemophilus influenzae is one of the major pathogenic bacteria causing paediatric infectious disease. Encapsulated *H. influenzae* strains are distinguished from non-typeable (NT) strains by the presence of one of six structurally and serologically distinct polysaccharide capsules, referred to as serotypes a–f. It is well-known that *H. influenzae* serotype b strains (Hib), which have high virulence compared to non-serotype b and NT strains, constitute a major cause of invasive infections such as meningitis, sepsis and epiglottitis in children [1]. Recently, a PCR method has been devised for serotyping *H. influenzae* strains, and was shown to have high sensitivity and specificity compared with the traditional slide agglutination test (SAT) [2].

The *H. influenzae* capsulation locus *cap* is a DNA fragment of *c.* 1.7 kb that is common to all strains that are typeable on the basis of their capsular structure. It consists of three regions (regions 1–3). All serotypeable strains share regions 1 and 3, but differ in region 2, which is considered to be a type-specific region. Most Hib strains possess two copies of *cap*, namely an intact copy and a second copy that has lost 1.2 kb of flanking region containing the *bexA* gene, which is necessary for capsule export [3,4]. A pair of primers (HI-1 and HI-2) has been designed to amplify the *bexA* gene, and these can be used to differentiate capsular strains from NT strains [5]. In addition, it has been reported that Hib *cap* can be duplicated or reduced in the flanking region containing the *bexA* gene during a homologous recombination process. Strains that lose the *bexA* gene cannot be identified by SAT, as they are unable to express the capsular polysaccharide. Similarly, they cannot be identified with primers HI-1 and HI-2, but can be detected by PCR with primers specific for type b [5].

In the USA, Hib vaccine was introduced in 1988 and has been used widely since 1990. As a consequence, the prevalence of infectious diseases caused by Hib has decreased dramatically, while a trend towards an increase in the incidence of diseases caused by NT *H. influenzae* strains or those belonging to other capsular serotypes has

Corresponding author and reprint requests: N. Ishiwada, Chiba 260-8670, Chuo-ku, Inohana, 1-8-1, Japan
E-mail: ishiwada@faculty.chiba-u.jp

been noted [6,7]. In contrast, the Hib vaccine has not yet been introduced in Japan. Thus, *H. influenzae* is still a leading cause of paediatric invasive infections, especially meningitis, in Japan [8]. In addition, paediatric invasive infections associated with non-type b and NT *H. influenzae* strains have not been well-documented. Therefore, it is necessary to determine the precise incidence rate of Hib and other encapsulated or NT *H. influenzae* strains by a technique that yields unequivocal results. The present study presents the results of a comparative serotypic characterisation by PCR [2,5] and SAT of *H. influenzae* isolates recovered from Japanese paediatric patients with invasive infection.

MATERIALS AND METHODS

Bacterial isolates

From January 2000 to May 2003, 53 isolates of *H. influenzae* were recovered from various sites of 53 paediatric patients with invasive infections. The patients were admitted either to the Department of Paediatrics of Chiba University Hospital or to ten other hospitals located in various areas throughout Japan. The isolates were from cerebrospinal fluid in cases of meningitis, or from blood. The isolates were stored at −80°C until they were investigated.

Bacterial identification

All isolates were identified as *H. influenzae* by standard laboratory methods on the basis of colony morphology, Gram's stain, X- and V-factor requirement, and negative porphyrin tests. The following six strains were used as positive controls for serotypes a, b, c, d, e and f, respectively: ATCC 9006, ATCC 10211, ATCC 9007, ATCC 9332, ATTC 8142 and ATCC 9833. A further five strains, namely *Staphylococcus aureus* ATCC 25923, *H. influenzae* ATCC 49766 (NT), *Moraxella catarrhalis* (clinical isolate), *Streptococcus* sp. (clinical isolate) and *Streptococcus pneumoniae* (clinical isolate), were used as negative controls.

Serotype determination by slide agglutination

Sub-cultures (20–24 h) of the *H. influenzae* isolates were tested initially with a commercially available slide agglutination kit for type b (Phadebact *Haemophilus* test; Pharmacia, Tokyo, Japan). Non-serotype b strains were then investigated further by counter-immunoelectrophoresis with antisera specific for serotypes a–f (Denka Seiken, Tokyo, Japan).

Serotype determination by PCR

Bacterial DNA was prepared by a modification of the methods described by LaClaire *et al.* [2] and Falla *et al.* [5]. Rapid DNA extraction was performed by resuspending a sweep of eight *H. influenzae* colonies (from an 18–20-h subculture on chocolate agar) in 25 μL of sterile distilled water and boiling for 3 min. The cell debris was pelleted by centrifugation at 2000 *g* for 2 min, and 20 μL of supernatant was stored at −20°C until required. Primers HI-1 and HI-2 [5] were used to detect the *bexA* gene. In addition, six pairs of type-specific primers (a1, a2; b1, b2; c1, c2; d1, d2; e1, e2; f1, f2) were used to detect serotypes a–f, respectively [2]. In a further experiment, all the NT isolates which gave negative results in the first round of PCR were subjected to PCR with type-specific primers in order to detect any capsule-deficient mutants of encapsulated strains. PCR amplification of target DNA was done with the conditions described previously [2,5], with resolution of amplification products on agarose 2% w/v gels and visualisation by staining with ethidium bromide. Fragment sizes of amplicons were compared with those of the positive controls and DNA micromaker size standards (E840; Amresco, Solon, OH, USA).

RESULTS

The capsular-specific primers HI-1 and HI-2 amplified DNA sequences of *c.* 400 bp from 48 of the 53 isolates. The 48 capsule-positive strains were analysed further with the six pairs of serotype-specific primers. Serotype b-specific bands were detected with 47 of these 48 isolates. The remaining isolate was positive for type e. None of the five NT isolates yielded amplification products with the serotype-specific primers. This finding indicated that none of these five NT isolates possessed a capsulation locus. Only one discrepancy was found between the results obtained with the SAT and PCR assays, in that one isolate was identified as NT with the SAT, but as serotype e with the PCR method.

Table 1 shows the correlation between the invasive *H. influenzae* isolates and the age, sex and clinical diagnosis of the patients. Total isolation rates were 45.3% in males and 54.7% in females. Most (75.5%) isolates were from patients aged <3 years. The serotype e isolate was from

Table 1. Distribution of isolates according to the age, sex and clinical diagnosis of patients

Patient characteristics/diagnosis	Type b (*n* = 47)	Type e (*n* = 1)	Non-typeable (*n* = 5)	Total (*n* = 53)
Sex				
Male	23	0	1	24
Female	24	1	4	29
Age (years)				
< 1	12	0	3	15
1–2	24	0	1	25
3–5	11	0	1	12
> 6	0	1	0	1
Clinical diagnosis				
Meningitis	39	0	2	41
Sepsis	2	1	0	3
Septic arthritis	3	0	0	3
Epiglottitis	3	0	0	3
Septic pneumonia	0	0	3	3

the blood of a 14-year-old female with sepsis. In total, 41 (77.4%) patients had meningitis, three had epiglottitis, three had septic arthritis, three had sepsis, and three had pneumonia with septicaemia. Among the 41 isolates from patients with meningitis, 39 (95.1%) were Hib and two (4.9%) were NT *H. influenzae*. Of the five patients with invasive infection caused by NT *H. influenzae*, two had meningitis and three had pneumonia with septicaemia. In addition, three of the five NT isolates were from patients aged < 1 year.

DISCUSSION

Before the introduction of Hib conjugate vaccine, serotype b *H. influenzae* was the leading cause of bacterial meningitis and a common cause of other invasive infections in infants and young children. Several studies conducted in the USA, Ireland, Brazil and South Korea showed that most *H. influenzae* strains that caused invasive infection were type b, while NT strains, especially encapsulated non-type b strains, were uncommon [9–12]. However, following the introduction of Hib conjugate vaccine, the incidence of Hib invasive infections has decreased dramatically [13]. As a consequence, invasive infections caused by encapsulated non-type b and NT strains have become relatively more frequent [14,15].

Conventionally, serotyping of *H. influenzae* strains has been performed by standard SATs [16]. However, these tests have been shown to be unreliable, and a PCR serotyping method, with high sensitivity and specificity compared with the standard SAT, has now become available [2,5]. The usefulness of this technique for typing *H. influenzae* has already been confirmed in several studies [14,17], and the technique has been used to investigate the incidence of invasive infections caused by capsule-deficient mutant Hib strains (b^- strains) [18,19]. It is likely that the single discrepant result in the present study was caused by the low sensitivity of the antiserum, resulting in difficulties in interpreting the SAT.

In the present study, all 53 isolates were examined with the PCR method. With regard to disease spectrum, the present study indicated that meningitis cases accounted for 77% of all the cases of invasive disease caused by Hib. The remaining 23% of cases involved epiglottitis, septic arthritis, sepsis and pneumonia. This distribution is similar to that reported in the USA in the pre-vaccination era [20]. Information on the incidence of invasive *H. influenzae* infections among Japanese children has previously been limited. According to a nationwide survey of *H. influenzae* meningitis, the incidence is 4.7 cases/100 000/year [8]. Based on these data, the estimated number of patients with meningitis in the entire country during the study period was *c.* 1900, so that the 41 cases in the present study represented 2.2% of all the cases of meningitis caused by *H. influenzae* in Japan. Not surprisingly, most (88.7%) of the invasive *H. influenzae* isolates were of serotype b. This finding is consistent with the data reported previously by many investigators from other countries in the pre-vaccination era [9–12].

The present study also confirmed the incidence of invasive infections caused by NT *H. influenzae*, which accounted for 9.4% of the total. According to previous reports, some NT *H. influenzae* strains show evidence of capsulation sequences, suggesting that a subgroup of NT strains is derived from an encapsulated precursor. Such strains might be generated by loss of their capsulation ability through homologous recombination [19], but the present study did not identify any invasive capsule-deficient mutants of encapsulated strains. Although the number of isolates studied was too small to be conclusive, the findings suggested that the incidence of invasive b^- strains of *H. influenzae* is extremely low in Japanese paediatric infections.

Although the Hib conjugate vaccine has not yet been introduced in Japan, this type of vaccine may be used widely in the near future. As a result, other encapsulated non-type b and NT strains may then emerge as a cause of invasive infection in the post-vaccination era. Therefore, it is necessary to continue *H. influenzae* surveillance to detect any alteration in disease-causing *H. influenzae* in the community, and the information acquired from such surveillance studies should be exploited to institute effective vaccination strategies. The PCR-based method, with its high sensitivity and specificity, should be valuable for serotyping invasive *H. influenzae* strains in Japan. As suggested previously [17], although it would be difficult to introduce this technique into all clinical laboratories, its use should be encouraged in reference laboratories as an accurate means of monitoring *H. influenzae* isolates recovered from invasive infections.

REFERENCES

1. Ward JI, Zangwill KM. *Haemophilus influenzae*. In: Feigin RD, Cherry JD, eds. Textbook of pediatric infectious diseases, 4th edn. Philadelphia: Saunders, 1998; 1465–1482.
2. LaClaire LL, Tondella MLC, Beall DS *et al*. Identification of *Haemophilus influenzae* serotypes by standard slide agglutination serotyping and PCR-based capsule typing. *J Clin Microbiol* 2003; **41**: 393–396.
3. Kroll JS. The genetics of encapsulation in *Haemophilus influenzae*. *J Infect Dis* 1992; **165**(suppl 1): S93–S96.
4. Kroll JS, Zamze S, Loynds B, Moxon ER. Common organization of chromosomal loci for production of different capsular polysaccharides in *Haemophilus influenzae*. *J Bacteriol* 1989; **171**: 3343–3347.
5. Falla TJ, Crook DWM, Brophy LN *et al*. PCR for capsular typing of *Haemophilus influenzae*. *J Clin Microbiol* 1994; **32**: 2382–2386.
6. Adams WG, Deaver KA, Cochi SL *et al*. Decline of childhood *Haemophilus influenzae* type b (Hib) disease in the Hib vaccine era. *JAMA* 1993; **269**: 221–226.
7. Ogilvie C, Omikunle A, Wang Y *et al*. Capsulation loci of non-serotype b encapsulated *Haemophilus influenzae*. *J Infect Dis* 2001; **184**: 144–149.
8. Kamiya H, Uehara S, Kato T *et al*. Childhood bacterial meningitis in Japan. *Pediatr Infect Dis J* 1998; **17**: S183–S185.
9. Wenger JD, Pierce R, Deaver K *et al*. Invasive *Haemophilus influenzae* disease: a population-based evaluation of the role of capsular polysaccharide serotype. *J Infect Dis* 1992; **165**(suppl 1): S34–S35.
10. Mononey AC, Fogarty J, Clarke P *et al*. Invasive *Haemophilus influenzae* in the Republic of Ireland. *Eur J Clin Microbiol Infect Dis* 1997; **16**: 377–380.
11. Zanella RC, Casagrande ST, Bokermann S *et al*. Characterization of *Haemophilus influenzae* isolated from invasive disease in Brazil from 1990 to 1999. *Microb Drug Resist* 1990; **8**: 67–72.
12. Kwak YH, Jung HS, Park SE *et al*. Serotypes and antimicrobial susceptibility in clinical isolates of *Haemophilus influenzae* from Korean children in prevaccination era. *J Korean Med Sci* 2000; **15**: 616–622.
13. Schuchat A, Robinson K, Wenger JD *et al*. Bacterial meningitis in the United States in 1995. *N Engl J Med* 1997; **337**: 970–976.
14. Ribeiro GS, Reis JN, Cordeiro SM *et al*. Prevention of *Haemophilus influenzae* type b (Hib) meningitis and emergence of serotype replacement with type a strains after introduction of Hib immunization in Brazil. *J Infect Dis* 2003; **187**: 109–116.
15. Heath PT, Booy R, Azzopardi HJ *et al*. Non-type b *Haemophilus influenzae* disease: clinical and epidemiologic characteristics in the *Haemophilus influenzae* type b vaccine era. *Pediatr Infect Dis J* 2001; **20**: 300–305.
16. Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH, eds. *Haemophilus*. In: *Manual of clinical microbiology*, 7th edn. Washington DC: American Society for Microbiology, 1999; 604–613.
17. Bokermann S, Zanella RC, Lemos APS *et al*. Evaluation of methodology for serotyping invasive and nasopharyngeal isolates of *Haemophilus influenzae* in the ongoing surveillance in Brazil. *J Clin Microbiol* 2003; **41**: 5546–5550.
18. Falla TJ, Dobson SM, Crook DWM *et al*. Population-based study of non-typeable *Haemophilus influenzae* invasive disease in children and neonates. *Lancet* 1993; **341**: 851–854.
19. Cerquetti M, Atti MLC, Renna G *et al*. Characterization of non-type b *Haemophilus influenzae* strains isolated from patients with invasive disease. *J Clin Microbiol* 2000; **38**: 4649–4652.
20. Dajani AS, Asmar BI, Thirumoorthi MC. Systemic *Haemophilus influenzae* disease: an overview. *J Pediatr* 1979; **94**: 355–364.